FORM PTO-1390 (Modified) (REV 11-2000) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER	U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR
221609US0PCT	10/089986

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/JP00/06527	22 September 2000	6 October 1999

TITLE OF INVENTION

GENE ENCODING PROTEIN CAPABLE OF REGENERATING LUCIFERIN, NOVEL RECOMBINANAT DNA, AND PROCESS FOR PRODUCING PROTEIN CAPABLE OF REGENERATING LUCIFERIN

APPLICANT(S) FOR DO/EO/US

KUROSAWA Keiko et al.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (24) indicated below.
4. ☒ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
   a. ☐ is attached hereto (required only if not communicated by the International Bureau).
   b. ☒ has been communicated by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. ☒ is attached hereto.
   b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
10. ☐ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).
11. ☐ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☒ A copy of the International Search Report (PCT/ISA/210).

Items 13 to 20 below concern document(s) or information included:

13. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☒ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
20. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
21. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
22. ☐ Certificate of Mailing by Express Mail
23. ☒ Other items or information:

PCT/IB/308
Form PTO-1449
Request for Priority
Preliminary Amendment (with sequence listing and computer readable sequence listing)

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
10/089986	PCT/JP00/06527	221609US0PCT

24. The following fees are submitted:	CALCULATIONS PTO USE ONLY	
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :		
☐ Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$1040.00**		
☒ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$890.00**		
☐ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$740.00**		
☐ International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$710.00**		
☐ International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) **$100.00**		
ENTER APPROPRIATE BASIC FEE AMOUNT =	**$890.00**	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).	**$0.00**	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	18 - 20 =	0	x $18.00	**$0.00**	
Independent claims	4 - 3 =	1	x $84.00	**$84.00**	
Multiple Dependent Claims **(check if applicable)**.			☐	**$0.00**	
TOTAL OF ABOVE CALCULATIONS =				**$974.00**	
☐ Applicant claims small entity status. See 37 CFR 1.27). The fees indicated above are reduced by 1/2.				**$0.00**	
SUBTOTAL =				**$974.00**	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)). +				**$0.00**	
TOTAL NATIONAL FEE =				**$974.00**	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) **(check if applicable).** ☐				**$0.00**	
TOTAL FEES ENCLOSED =				**$974.00**	
				Amount to be: refunded	$
				charged	$

a. ☒ A check in the amount of **$974.00** to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **15-0030** A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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22850

Surinder Sachar
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Norman F. Oblon
NAME

24,913
REGISTRATION NUMBER

April 8 2002
DATE

Docket No. 221609US0PCT

IN THE UNITED STATES PATENT & TRADEMARK OFFICE

IN RE APPLICATION OF: : ATTN: NEW APPLICATIONS

KEIKO KUROSAWA ET AL :

SERIAL NO. NEW PCT APPLICATION
(Based on PCT/JP00/06527) :

FILED: HERWITH :

FOR:GENE ENCODING PROTEIN CAPABLE OF REGENERATING LUCIFERIN, NOVEL RECOMBINANT DNA, AND PROCESS FOR PRODUCING PROTEIN CAPABLE OF REGENERATING LUCIFERIN

PRELIMINARY AMENDMENT

ASSISTANT COMMISSIONER FOR PATENTS
WASHINGTON, D.C. 20231

SIR:

Prior to Examination on the merits, please amend the above-identified application as follows.

IN THE SPECIFICATION

Please amend the specification as follows.

Page 18 (Abstract), after the last line, beginning on a new page, please replace the original Sequence Listing with the attached substitute Sequence Listing.

IN THE CLAIMS

Cancel Claims 1-11 and add the following claims:

12. An isolated polynucleotide, which encodes a protein that regenerates luciferin from oxyluciferin and D-cysteine.

13. The isolated polynucleotide of claim 12, which is from a luminescent organism.

14. The isolated polynucleotide of Claim 13, wherein the luminescent organism is of the genus Coleoptera.

15. The isolated polynucleotide of Claim 13, wherein the luminescent organism is a firefly.

16. The isolated polynucleotide of claim 13, where the luminescent organism is a North American firefly.

17. The isolated polynucleotide of claim 13, wherein the luminescent organism is an American firefly.

18. An isolated polynucleotide encoding a protein comprising an amino acid sequence of SEQ ID NO:2 or a protein having a deletion, mutation, substitution, or addition of one or more amino acids, and which can regenerate luciferin.

19. An isolated polynucleotide which is at least 50% homologous to the amino acid sequence of SEQ ID NO:2 and which encodes a protein that can regenerate luciferin.

20. A vector comprising the isolated polynucleotide of Claim 12.

21. A vector comprising the isolated polynucleotide of Claim 18.

22. A vector comprising the isolated polynucleotide of Claim 19.

23. A host cell comprising the isolated polynucleotide of Claim 12.

24. A host cell comprising the isolated polynucleotide of Claim 18.

25. A host cell comprising the isolated polynucleotide of Claim 19.

26. A method of producing a protein that can regenerate luciferin, comprising culturing the host cell of claim 23 in a medium, and collecting the protein

27. A method of producing a protein that can regenerate luciferin, comprising

culturing the host cell of claim 24 in a medium, and collecting the protein.

28. A method of producing a protein that can regenerate luciferin, comprising culturing the host cell of claim 25 in a medium, and collecting the protein.

29. *Escherichia coli* FERM BP6908.

REMARKS

Claims 12-29 are pending in the present application. Claims 12-28 are supported by Claims 1-11.

Applicants have now submitted a substitute Sequence Listing and a corresponding computer-readable Sequence Listing. The sequence information recorded in the corresponding computer-readable Sequence Listing is identical to the paper copy of the Sequence Listing. Support for all of the sequences listed in the Sequence Listing is found in the present application as originally filed, SEQ ID NO:1 is supported by the deposited microorganism containing the cloned gene of the luciferin regenerating protein, described on page 13. No new matter is believed to have been introduced by the submission of the substitute Sequence Listing and the corresponding computer-readable Sequence Listing.

Applicants submit that the present application is ready for examination on the merits. Early notice to this effect is earnestly solicited.

Respectfully submitted,

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SPECIFICATION

TITLE OF THE INVENTION

GENE ENCODING PROTEIN CAPABLE OF REGENERATING LUCIFERIN, NOVEL RECOMBINANT DNA, AND PROCESS FOR PRODUCING PROTEIN CAPABLE OF REGENERATING LUCIFERIN

TECHNICAL FIELD

The present invention relates to a gene encoding a protein capable of regenerating luciferin, a novel recombinant DNA, and process for producing a protein capable of regenerating luciferin.

BACKGROUND ART

Luciferin is a substrate of a bioluminescence enzyme, luciferase, and after emitting light as a result of luciferase reaction, is converted to oxyluciferin. ATP measurement methods using luciferase are widely used in the fields of medical science and food hygiene. However, luciferin which is used as a substrate, is expensive and the luciferase reaction is inhibited by oxyluciferin produced after reaction. Thus, removal of oxyluciferin or regeneration to luciferin will enable further development of the ATP measurement methods using luciferase. A protein which is derived from a firefly and capable of regenerating luciferin from oxyluciferin has been found (U.S. Pat. No. 5814504). However, only a small quantity of the protein

can be extracted from a firefly body so that industrial application of the protein has been difficult.

Addition of such a protein capable of regenerating luciferin to the luciferin-luciferase reaction system enables improvement in durability of luminescence and reduction in the amount of luciferase and luciferin to be used.

DISCLOSURE OF THE INVENTION

The object of the present invention is to provide a process for producing a protein capable of regenerating luciferin using a recombinant DNA to which a gene encoding the protein capable of regenerating luciferin has been inserted.

As a result of dedicated research on the above object, we have succeeded in isolating a gene which is derived from an insect belonging to the class Coleoptera and encodes a protein capable of regenerating luciferin, determining the gene structure, and obtaining a recombinant DNA by inserting a gene encoding a protein capable of regenerating luciferin into a vector DNA. Then we have completed the present invention by finding that a protein capable of regenerating luciferin can be produced efficiently by culturing a transformant or a transductant wherein the recombinant DNA is contained in a host cell.

That is, a first invention of the present invention is a gene which encodes a protein capable of regenerating luciferin by acting on oxyluciferin and D-cysteine.

A second invention of the present invention is the above gene which is derived from an organism capable of luminescence.

A third invention of the present invention is a gene which encodes the following protein (a) or (b):

(a) a protein which comprises an amino acid sequence represented by SEQ ID NO: 2;

(b) a protein which comprises an amino acid sequence derived from the amino acid sequence (a) by deletion, substitution, or addition of one or more amino acids, and is capable of regenerating luciferin.

A forth invention of the present invention is a gene which has 50% or more homology with the amino acid sequence represented by SEQ ID NO: 2 and encodes a protein capable of regenerating luciferin.

A fifth invention of the present invention is a novel recombinant DNA which is characterized in that the above gene encoding a protein capable of regenerating luciferin is inserted into vector DNA.

A sixth invention of the present invention is a transformant or a transductant which comprises the above recombinant DNA.

A seventh invention of the present invention is a process for producing a protein capable of regenerating luciferin which comprises culturing the above transformant or transductant in

a medium and collecting the protein capable of regenerating luciferin from the culture product.

Hereinafter, the present invention is described in detail.

The gene of the present invention which encodes a protein capable of regenerating luciferin is obtained from a Coleoptera.

For example, the gene of the present invention which encodes a protein capable of regenerating luciferin can be obtained as follows.

First, mRNA is extracted from the luminous organ of an American firefly.

Next, synthetic DNA is prepared based on an amino acid sequence of a purified protein capable of regenerating luciferin and the codon frequency of an American firefly. Then a reverse transcription polymerase chain reaction (hereinafter abbreviated as a RT-PCR method) is performed using the mRNA obtained above as a template, thereby obtaining DNA encoding a part of the protein capable of regenerating luciferin.

cDNA is synthesized from the mRNA obtained above using reverse transcriptase. Then the cDNA, as an intact cDNA or as an amplified gene encoding a protein capable of regenerating luciferin by the PCR method, is incorporated into a vector DNA by standard techniques. Examples of a vector DNA used herein include a plasmid DNA, such as pUC19(Takara Shuzo), pBR322

(Takara Shuzo), pBluescript SK+ (Stratagene), and pMAL-C2 (NEW England Labs), and bacteriophage DNA, such as λ ENBL3 (Stratagene) and λ DASH II (Funakoshi). The obtained recombinant DNA is transformed or transduced into, for example, *Escherichia coli* K-12, preferably *Escherichia coli* JM109 (Toyobo), DH5α (Toyobo) or XL1-Blue (Funakoshi), thereby obtaining transformants or transductants, respectively. In addition to the above, examples of a host cell used herein include bacteria, such as *Escherichia coli* other than *E. coli* K-12, yeast, mold, Actinomycetes, silk worms, and animal cells.

Transformation can be performed by, for example D. M. Morrison's method (Method in Enzymology, 68, 326-331, 1979). Transduction can be performed by, for example B. Hohn's method (Method in Enzymology, 68, 299-309, 1979).

A novel recombinant DNA which is purified from the above transformant or transductant can be obtained by, for example, P. Guerry et al.'s method [J. Bacteriology, vol. 116, 1064-1066 (1973)] and D. B. Clewell's method [J. Bacteriology, vol. 110, 667-676 (1972)].

Further, the entire nucleotide sequence of a gene which encodes a protein capable of regenerating luciferin is analyzed (see SEQ ID NO: 1) using DNA comprising the above gene which encodes the protein capable of regenerating luciferin and a 373A DNA sequence system (Perkin-Elmer) indicated in the later described Example (9). Then, the primary sequence of amino acids of a polypeptide which is translated by a gene comprising the

above nucleotide sequence is determined (see SEQ ID NO: 2).

Further, the present invention encompasses any gene which encodes a protein capable of regenerating luciferin comprising an amino acid sequence which is derived from the amino acid sequence of SEQ ID NO: 2 by deletion, substitution, or addition of one or more, preferably several amino acids and is capable of regenerating luciferin.

Furthermore, the present invention encompasses any gene which encodes a protein having a 50% or more homology with the amino acid sequence of SEQ ID NO: 2 and capable of regenerating luciferin.

Any method can be employed to obtain a gene which encodes a protein capable of regenerating luciferin comprising an amino acid sequence which is derived from the amino acid sequence of SEQ ID NO: 2 by deletion, substitution or addition of one or more amino acids and is capable of regenerating luciferin. Examples of such a method include site-directed mutagenesis which is a known technique to cause point mutation or deletion to occur in a gene, a method which involves selective cleavage of a gene, removal or addition of a selected nucleotide, and ligation of the gene, and an oligonucleotide mutation induction method.

A protein capable of regenerating luciferin can be produced as described below using a transformant or a transductant capable of regenerating luciferin obtained as described above, for example, a strain belonging to the genus *Escherichia*. The above

microorganism may be cultured by a normal solid culture method, preferably a liquid culture method.

A medium used for culturing the above microorganism is supplemented with, for example, one or more types of nitrogen source, such as yeast extract, Peptone, meat extract, corn steep liquor, or exudates of soybean or wheat koji; and one or more types of inorganic salt, such as potassium dihydrogenphosphate, dipotassium hydrogenphosphate, magnesium sulfate, ferric chloride, ferric sulfate or manganese sulfate, and if necessary, appropriately supplemented with carbohydrate material, vitamin and the like.

The initial pH of a medium is appropriately adjusted to 7 to 9. Culturing is performed at 30℃ to 42℃, preferably at around 37℃ for 6 to 24 hours by aeration (agitation) - submerged culture, shaking culture, stationary culture or the like. After culturing, a protein capable of regenerating luciferin can be collected from the culture product by normal techniques for collecting enzymes.

Cells were isolated from the culture product by a technique, such as filtration or centrifugation, and washed. The protein capable of regenerating luciferin is preferably collected from cells. In this case, intact cells can be used. Preferably, the protein capable of regenerating luciferin is collected from cells by, for example, a method which disrupts cells using various disruptive means, such as an ultrasonicator, french press or Dyno-Mill, a method which digests cell walls using a cell wall

digesting enzyme, such as lysozyme, and a method which extracts enzyme from the cell using a surfactant, such as Triton X-100.

The protein capable of regenerating luciferin can be isolated from the thus obtained crude solution of protein having ability to regenerate luciferase by a standard technique for enzyme purification. Preferably performed is an appropriate combination of such techniques including ammonium sulfate salting out technique, precipitation technique using organic solvents, ion exchange chromatography, gel filtration chromatography, adsorption chromatography and electrophoresis.

The obtained protein capable of regenerating luciferin can regenerate luciferin by acting on oxyluciferin and D-cysteine.

(Method for measuring ability to regenerate luciferin)

(Reagent)

A 0.1 mM oxyluciferin

B 0.01 mM D-cysteine

C 25 mM glycylglycine + 5.4mM magnesium sulfate

D 10 mM ATP (pH7.8)

E 5 mg/ml luciferase

(Procedure)

1. Prepare a mixed solution of the following reagents.

0.005 ml A

0.010 ml B

0.085 ml C

2. Add 0.01 ml of the protein solution and allow to react at 37℃ for a certain time.

3. Mix 0.01 ml of the reaction solution with 0.1 ml of C.

4. Prepare a luciferase mixed solution of the following reagents.

10 ml D

1 ml E

5. Add 0.1 ml of the mixed solution of 4 to that of 3, and then measure the amount of light emitted using a luminometer.

Best mode for carrying out the invention

Hereinafter, the present invention is described in more detail by Examples.

EXAMPLES

[Example 1]

(1) Preparation of American firefly mRNA

The tail portion of American firefly (Sigma) 10 g disrupted with a mortar and a pestle was suspended in 10 ml of ISOGEN (Wako Pure Chemical Industries, Ltd.), a reagent for extracting RNA, and then centrifuged at 2700 r.p.m. for 5 min, thereby obtaining RNA fraction. From the fraction, 0.51 mg of mRNA was obtained according to the method described in the Current Protocols in Molecular Biology (WILEY Interscience, 1989).

(2) Synthesis of primer

Approximately 10 μg of the protein capable of regenerating luciferin purified in (1) was subjected to a protein sequencer (Perkin-Elmer Corporation), so that the N-terminal amino acid sequence was determined. Further, approximately 10 μg of the

protein capable of regenerating luciferin purified in (1) was digested with trypsin. Then 6 peptides obtained with HPLC were subjected to a protein sequencer, so that the internal amino acid sequence was determined. Furthermore, the codon frequency of American firefly was examined. Based on this information, primers shown in SEQ ID NOS: 3 and 4 were synthesized by Amersham Pharmacia Biotech's entrusted custom synthesis.

(3) RT-PCR

A reaction solution was prepared to have the following composition, and a reverse transcription reaction was allowed to proceed for 30 min at 42℃. Then, denaturation was performed at 99℃ for 5 min, and then stored at 5℃.

(Composition of reaction solution)

Magnesium chloride	5 mM
*10xRNA PCR buffer	2 μl
water	8.5 μl
dNTP	1 mM each
RNase inhibitor	1 U/μl
*AMV reverse transcriptase XL	0.25 U/μl
*oligo dT adapter primer	0.125 μM
mRNA	1 μg

*manufactured by Takara Shuzo

Next, 80 μl of the reaction solution prepared to have the following composition was added to a tube in which reverse transcription had been performed. Then PCR was performed under a reaction condition for 30 cycles, each consisting of

denaturation at 94℃ for 30 sec, annealing at 62℃ for 30 sec, and elongation reaction at 72℃ for 1.5 min.

(Composition of reaction solution)

Primer (SEQ ID NO: 3)	0.2 μM
Primer (SEQ ID NO: 4)	0.2 μM
*10xRNA PCR buffer	8 μl
Magnesium chloride	2.5 mM
*Taq polymerase	2.5 Unit

Water (add water to a final volume of 80 μl)

*manufactured by Takara Shuzo

After PCR, the reaction solution was subjected to agarose gel electrophoresis, so that a band of approximately 0.75kb regarded as a target fragment was confirmed. The band was cut out and purified with GENECLEAN II (BIO 101).

(4) Determination and analysis of nucleotide sequence of purified DNA fragment

The nucleotide sequence of the purified DNA fragment was determined and analyzed using a 373A DNA sequence system (Perkin-Elmer). Thus, an amino acid sequence which had been deduced from the determined nucleotide sequence was confirmed to comprise the previously described amino acid sequence (His Glu Thr Gln Thr Leu Tyr Phe Val Asp Thr). Thus, a partial sequence of the gene which encodes a protein capable of regenerating luciferin was confirmed to be present in the DNA fragment amplified by the above RT-PCR.

(5) Analysis of downstream region by 3'RACE

First, a primer was designed according to the above analysis for DNA sequence, and then synthesized by Amersham Pharmacia Biotech (SEQ ID NO: 5). RT-PCR was performed using the primer, the above mRNA and 3'-Full RACE CoreSet (Takara Shuzo), thereby amplifying 3' unknown region. The reaction solution was subjected to agarose electrophoresis, a DNA fragment of approximately 650 bp was purified and extracted with RecoChip (Takara Shuzo), and the nucleotide sequence was determined and analyzed using a DNA sequencer. Therefore, the 5' region of the determined nucleotide sequence was confirmed to contain a sequence being the same as that of the 3' sequence of the partial sequence of the above gene encoding a protein capable of regenerating luciferin. Further, an amino acid sequence which had been deduced from the determined nucleotide sequence was confirmed to comprise the previously described amino acid sequence (Ile Pro Asp Pro Gln Val Thr Ser Val Ala Phe Gly Gly Pro Asn Leu Asp Glu).

(6) Analysis of upstream region by 5' RACE

First, primers were designed according to the above analysis for DNA sequence, and then synthesized by Amersham Pharmacia Biotech (SEQ ID NOS: 6 to 9). RT-PCR was performed using the primers, the above mRNA and 5'-Full RACE CoreSet (Takara Shuzo), thereby amplifying 5' unknown region. The reaction solution was subjected to agarose electrophoresis, a DNA fragment of approximately 400 bp was purified and extracted with RecoChip (Takara Shuzo), and the nucleotide sequence was determined and analyzed using a DNA sequencer. Therefore, the determined

nucleotide sequence was confirmed to contain a sequence being the same as that of the partial sequence of the above gene encoding a protein capable of regenerating luciferin. Further, an amino acid sequence which had been deduced from the determined nucleotide sequence was confirmed to comprise the previously described amino acid sequence (Gly Pro Val Val Glu Lys Ile Ala Glu Leu Gly Lys).

(7) Recovery of gene fragment by RT-PCR

A translation initiation codon and a termination codon were deduced from the above three nucleotide sequences, and then the primer DNAs of the N terminal region and the C terminal region were synthesized by Amersham Pharmacia Biotech (SEQ ID NOS: 10 and 11). RT-PCR was performed using the primers and the above mRNA, and then the reaction solution was analyzed by agarose electrophoresis. As a result, a band of approximately 900 bp was confirmed. A DNA fragment contained in the band was purified and extracted with a RecoChip (Takara Shuzo), followed by digestion with restriction enzymes *Eco* RI and *Pst* I (both manufactured by Takara Shuzo). Separately, a plasmid pKK223-3 (Pharmacia) was digested with restriction enzymes *Eco* RI and *Pst* I and purified by agarose electrophoresis. This was ligated to the above purified and extracted DNA fragment, and then transformation of *E. coli* JM109 (Toyobo) was performed. The transformant strain, *E. coli* JM109 (pLRE), was deposited to Patent and Bio-Resource Center, National Institute of Advanced Industrial Science and Technology as FERM BP-6908.

(8) Confirmation of activity

E. coli JM109 (pLRE) cells were shake-cultured to Klett 100 at 37℃ in 10 ml of TY medium (1% bacto trypton, 0.5% bactoyeast extract, 0.5% NaCl, pH 7.0) containing 50 μg/ml ampicillin. Then, IPTG was added to a final concentration of 1mM, followed by another 4 hours of culturing. The culture solution was treated 4 times (20 sec each) using an ultrasonicator (Ultrasonicgenerator, Nissei) while cooling on ice. The solution was put into an Eppendorf tube, and centrifuged at 12,000 r. p. m. with a micro centrifuge for 10 min, thereby separating into supernatant and precipitation fractions. The supernatant was transferred to another Eppendorf tube and ability thereof to regenerate luciferin was measured by the previously described method for measuring enzyme activity. While *E. coli* comprising only a vector had 0.94 kcount/ml, *E. coli* JM109 (pLRE) had 10.58 kcount/ml and was confirmed to be capable of regenerating luciferin.

(9) Analysis of gene encoding protein capable of regenerating luciferin

Confirmation of the luciferin regenerating ability of *E. coli* JM109 (pLRE) revealed that the insertion fragment of pLRE comprised the gene of the protein capable of regenerating luciferin. Then, the nucleotide sequence was determined for the plasmid DNA using a 373A DNA sequence system (Perkin-Elmer). The determined nucleotide sequence and an amino acid sequence of a polypeptide which is translated from the DNA sequence are shown in SEQ ID NO: 1 and SEQ ID NO: 2, respectively. The gene of the protein capable of regenerating luciferin had a coding region of 924 bp and encoded 308 amino acids.

INDUSTRIAL APPLICABILITY

The present invention is industrially very useful because the invention enables efficient production of a protein capable of regenerating luciferin.

CLAIMS

1. A gene which encodes a protein capable of regenerating luciferin by acting on oxyluciferin and D-cysteine.

2. The gene of claim 1 which is derived from an organism capable of luminescence.

3. The gene of claim 2 wherein the organism capable of luminescence is a Coleoptera.

4. The gene of claim 2 wherein the organism capable of luminescence is a firefly.

5. The gene of claim 2 wherein the organism capable of luminescence is a North American firefly.

6. The gene of claim 2 wherein the organism capable of luminescence is an American firefly.

7. A gene which encodes the following protein (a) or (b):

(a) a protein which comprises an amino acid sequence represented by SEQ ID NO: 2;

(b) a protein which comprises an amino acid sequence derived from the amino acid sequence (a) by deletion, substitution, or addition of one or more amino acids, and is capable of regenerating luciferin.

8. A gene which has a 50% or more homology with the amino acid sequence represented by SEQ ID NO: 2 and encodes a protein capable of regenerating luciferin.

9. A novel recombinant DNA which is characterized in that the gene of claims 1 to 8 encoding a protein capable of regenerating luciferin is inserted into a vector DNA.

10. A transformant or a transductant which comprises the

recombinant DNA of claim 9.

11. A method of producing a protein capable of regenerating luciferin which comprises culturing the transformant or transductant of claim 10 in a medium and collecting the protein capable of regenerating luciferin from the culture product.

ABSTRACT

A gene encoding a protein which is capable of regenerating luciferin by acting on oxyluciferin and D-cysteine and thus regenerating luciferin; the above gene originating in a luminous organism; a protein encoded by the above gene; and a process for producing a protein capable of regenerating luciferin characterized by comprising culturing a transformant or a transductant having the above gene transferred therein and collecting the protein capable of regenerating luciferin from the culture. Thus, the protein capable of regenerating luciferin can be efficiently produced, which brings about a great industrial advantage.

SEQUENCE LISTING

<110> KUROSAWA, Keiko
KAJIYAMA, Naoki

<120> GENE ENCODING PROTEIN HAVING THE ABILITY TO REGENERATE LUCIFERIN, NOVEL RECOMBINANT DNA, PROCESS FOR THE PREPARATION OF PROTEIN HAVING THE ABILITY TO REGENERATE LUCIFERIN

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Attorney's Docket No.:_____

DECLARATION, POWER OF ATTORNEY AND PETITION

I (We), the undersigned inventor(s), hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I (We) believe that I am (we are) the original, first, and joint (sole) inventor(s) of the subject matter which is claimed and for which a patent is sought on the invention entitled

GENE ENCODING PROTEIN CAPABLE OF REGENERATING LUCIFERIN, NOVEL RECOMBINANT DNA, AND PROCESS FOR PRODUCING PROTEIN CAPABLE OF REGENERATING LUCIFERIN

the specification of which

- ☐ is attached hereto.
- ☐ was filed on _____ as
  Application Serial No. _____
  and amended on _____.
- ■ was filed as PCT international application
  Number PCT/JP00/06527
  on September 22, 2000,
  and was amended under PCT Article 19
  on _____ (if applicable).

I (We) hereby state that I (We) have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above; that I (We) do not know and do not believe that this invention was ever known or used before my invention or discovery thereof, or patented or described in any printed publication in any country before my invention or discovery thereof, or more than one year prior to this application, or in public use or on sale in the United States for more than one year prior to this application; that this invention or discovery has not been patented or made the subject of an inventor's certificate in any country foreign to the United States on an application filed by me or my legal representatives or assigns more than twelve months before this application.

I (We) acknowledge the duty to disclose information known to be material to the patentability of this application as defined in Section 1.56 of Title 37 Code of Federal Regulations.

I (We) hereby claim foreign priority benefits under Section 119(a)-(d) of Title 35 United States Code, of any foreign application(s) for patent or inventor s certificate listed below and have also identified below any foreign application for patent or inventor s certificate having a filing date before that of the application on which priority is claimed:

Application No.	Country	Filing date	Priority claimed
285258/1999	Japan	October 6, 1999	■ Yes ☐ No
			☐ Yes ☐ No
			☐ Yes ☐ No
			☐ Yes ☐ No

I hereby claim the benefit under Section 119(e) of Title 35 United States Code, of any United States application(s) listed below.

(Application Number)	(Filing Date)
(Application Number)	(Filing Date)

I (We) hereby claim the benefit under Section 120 of Title 35 United States Code, of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Section 112 of Title 35 United States Code, I (We) acknowledge the duty to disclose material information as defined in Section 1.56(a) of Title 37 Code of Federal Regulations, which occurred between the filing date of the prior application and national or PCT international filing date of this application:

Application Serial No.	Filing Date	Status (pending, patented, abandoned)

And I (We) hereby appoint: Norman F. Oblon, Registration No. 24,618; Marvin J. Spivak, Registration No. 24,913; C. Irvin McClelland, Registration No. 21,124; Gregory J. Maier, Registration No. 25,599; Arthur I. Neustadt, Registration No. 24,854; Richard D. Kelly, Registration No. 27,757; James D. Hamilton, Registration No. 28,421; Eckhard H. Kuesters, Registration No. 28,870; Robert T. Pous, Registration No. 29,099; Charles L. Gholz, Registration No. 26,395; Vincent J. Sunderdick, Registration No. 29,004; William E. Beaumont, Registration No. 30,996; Robert F. Gnuse, Registration No. 27,295; Jean-Paul Lavalleye, Registration No. 31,451; Stephen G. Baxter, Registration No. 32,884; Martin M. Zoltick, Registration No. 35, 745; Robert W. Hahl, Registration No. 33,893; Richard L. Treanor, Registration No. 36, 379; Steven P. Weihrouch, Registration No. 32, 829; John T. Goolkasian, Registration No. 26, 142; Richard L. Chinn, Registration No. 34, 305; Steven E. Lipman, Registration No. 30, 011; Carl E. Schlier, Registration No. 34, 426; James J. Kulbaski, Registration No. 34, 648; Richard A Neifeld, Registration No. 35, 299; J. Derek Mason, Registration No. 35, 270; Surinder Sachar 34, 423; Christina M. Gadiano, Registration No. 37, 628; Jeffrey B. McIntyre, Registration No. 36, 867; and Paul E. Rauch, Registration No. 38, 591; our (my) attorneys, with full powers of substitution and revocation, to prosecute this application and to transact all business in the Patent Office connected therewith; and we (I) hereby request that all correspondence regarding this application be sent to the firm of OBLON, SPIVAK, McCLELLAND, MAIER & NEUSTADT, P.C. whose Post office address is: Fourth Floor, 1755 Jefferson Davis Highway, Arlington, Virginia 22202 U.S.A.

I (We) declare further that all statements made herein of my (our) knowledge are true and that all statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

1-00

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Signature of Inventor

Date

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Citizen of:

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